

Development and Validation of HPLC-MS² Methodology for the Accurate Determination of C4-C8 B-type Flavanols and Procyanidins

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Abstract

Cocoa flavanols and procyanidins (CFs), natural dietary bioactives, have been extensively studied over the past two decades. Human research on the safety and efficacy of CFs is critically dependent upon on the ability to reliably characterize the research materials that are utilized, and with growing consumer availability of CF-based products, reliable methods for the detection of potential adulteration are of increasing importance. The characterization of CF usually relies on the separation of the analytes by degree of polymerization followed by fluorescence detection, an approach that has recently been validated and accredited as an AOAC Official Method of Analysis (2020.05). More selective methods exploring mass spectrometry detection have been reported, but could not discuss method accuracy due to limited availability of analytical standards. This research focused on the development of a liquid chromatography-tandem mass spectrometry method (HPLC-MS²) using primary standards and synthetic ¹³C-labelled procyanidins as internal standards. The ability of MS² detection to discriminate A- and B-type procyanidins was demonstrated. Method performances were validated for degrees of polymerization up to four in seven food model matrices. Accuracy ranged from 90.9 to 125.4 % and precision was <10 % at lower concentration. Finally, the method was applied to cocoa-based samples and compared to AOAC 2020.05, supporting the use of NIST 8403 as reference material for LC-MS analysis.

Introduction

There is a growing scientific and consumer interest for plant-derived bioactives in the human diet as their intake is thought to mediate positive health effects. Examples of dietary bioactives include (poly)phenols such as flavanols and their polymeric derivatives, the procyanidins. Flavanols and procyanidins occur in many foods, and nearly always together, making the chemical analysis of these plant compounds in food often more challenging. In the context of the diet, foods such as ^{1,2}, apples, cocoa, nuts, grapes and berries ³ can be particularly rich in flavanols and PC. Specifically, cocoa flavanols (CFs) represent a particular group of flavanols and procyanidins that have been subjected to extensive research in recent years that has revealed beneficial effects on cardiovascular health and cognitive performance ⁴⁻⁷. In this context, developing accurate and reliable methods for the quantification of flavanols and procyanidins becomes essential in order to properly characterize materials used in human research and to better assess their levels and distribution in the diet. Furthermore, with growing consumer interest in CF-based products, the ability to reliably determine the authenticity of products, including the potential adulteration with other flavanol and procyanidin sources, is of increasing importance.

Analysis of the flavanol monomers is relatively straight forward ⁸, however, accurate quantitative analysis of procyanidins is a major challenge ⁹⁻¹¹. While there are four naturally occurring flavanol monomers, namely (+)- and (-)-epicatechin and (+)- and (-)-catechin, these monomers can form oligomeric procyanidins with a variety of configurations (Fig. 1). In addition, monomer sub-units can be linked via one or two covalent bonds, which gives rise to B-type and A-type procyanidins, respectively (Fig. 2; ¹²). B-type procyanidins are found in products such as cocoa and apples ¹³, in which monomeric units

predominantly have C4→C8 linkages (e.g. procyanidin B2), although C4→C6 bonds can also occur in less abundance (e.g. procyanidin B5) ^{1,2}. A-type procyanidins, that are found in peanuts and cranberries ¹³, C4→C8 links occur in conjunction with an O7→C2 ether bond (e.g. procyanidin A1 and A2). This complexity results in a large variety of procyanidins structures, which increases in parallel with the degree of polymerization (DP). In this context, LC-MS methodologies have been instrumental in identifying procyanidins of different sizes and structures, including oligomers containing other subunits such as gallocatechins and afzelechins ¹⁴. However, the use of LC-MS for the direct and accurate quantification of flavanols and procyanidins has not been reported and is dependent upon the the availability of analytical standards for method calibration and quantitative analysis. In this context, LC methods based on HILIC resolution have demonstrated the separation of a complex mixture of procyanidin based on DP, which simplifies the quantification by reducing the number of analytical standards required to one per DP. As a proof of the validity of this approach, a reliable and transferable method based on fluorescence detection was recently validated and later accredited by AOAC (2020.05) to quantify CF in cocoa-derived products ¹⁵. Racine et al. [16] reported the coupling of HILIC chromatography with tandem mass spectrometry detection and while this work constituted an important development of LC-MS for the quantitative analysis of procyanidins by degree of polymerisation, accuracy was not examined.

This paper reports the development and validation of a versatile HPLC-MS² method for the quantification of flavanols and procyanidins, using CFs as a model system. CFs were selected as their procyanidins have been extensively characterized, with the predominant structures being C4-C8 B-type oligomers ⁹. CF oligomeric fractions including DP2-7 isolated from the seeds of *Theobroma cacao* L. were used in combination with commercially-available procyanidins dimer standards, NIST cocoa extract reference material and, de novo chemically synthesized ¹³C-labeled procyanidins (Fig. 2) to i) develop HPLC-MS² conditions for cocoa procyanidins, ii) study MS² selectivity toward A-type and B-type procyanidins, iii) investigate potential matrix effects, and iv) for the validation and application to commercial samples.

Results And Discussion

Optimization of tandem mass spectrometry detection. Method development was initiated by studying collision induced dissociation (CID) of DP2-7 procyanidin standards isolated from cocoa (Table 1). The transformations identified on each target validate previously assigned fragments derived from complex mixtures analyzed by HPLC-MS² ¹³. Retro Deals-Alder, quinone methide formation and heterocyclic ring fission were observed in addition to the neutral losses of carbon dioxide and water. The identification of selective MS² transitions was the most challenging as B-type procyanidins present in cocoa show similar fragmentation patterns and interfering signals when forming multiple charged ions ¹⁶. In this context, two transitions were selected for each target procyanidin (Table 1). In order to optimize method selectivity, neutral losses of carbon dioxide and water were not considered for the development of quantitative MRM traces. The first transition was used as a quantitative trace and the second was used as a confirmation ion. These transitions were selected to maximize sensitivity and selectivity (Table 1).

Table 1

Collision induced dissociation (CID) with optimal cone voltage (CV) and collision energy (CE) for cocoa flavanols and procyanidins with degree of polymerization (DP) of 1-7.

Compound	Parent ions (m/z) (CV)	Fragments (m/z) observed under CID	Ions selected for MRM (m/z) (CE)
DP1	289 (15)	245 227 221 205 203 179 161 151 125 109	109 (26) 205 (16)
$^{13}\text{C}_3$ -DP1	292 (15)	248 125 109 223	109 (26)
DP2	577 (30)	559 451 425 407 381 289 287 245 125	425 (18) 451 (14)
$^{13}\text{C}_4$ -DP2	582 (30)	429 411 366 292 248 181 163 125	429 (26)
DP3	865 (45)	739 713 695 577 451 425 407 289 287 245 125	287 (32) 577 (24)
$^{13}\text{C}_4$ -DP3	869 (45)	529 455 410 291 125 244 343 428	291 (32)
DP4	1153 (45)	1108 956 865 821 577 425 413 407 381 289 287 245 125	287 (38) 575 (24)
$^{13}\text{C}_4$ -DP4	1158 (45)	581 291 413 743 870 1005 987 951	581 (24)
DP5	720 (45)	907 863 720 695 575 450 407 289 245 125	407 (28) 695 (20)
DP6	865 (50)	1151 577 450 425 407 289 245 125	577.5 (20) 1151 (16)
DP7	1008 (40)	1440 1312 1296 1151 865 863 788 701 577 425 946 413 407 389 287 245 125	946 (18) 1268 (26)

After the identification of transitions for targeted CFs, ^{13}C -labelled standards were included in the method. ^{13}C -labelled structures are shown in Fig. 1. When analyzed individually, ^{13}C labelled DP1-4 procyanidins did not interfere with the detection of procyanidins with natural isotope abundance and vice-versa. This was not the case for $^{13}\text{C}_4$ -DP5 as interferences were observed and were associated with the formation of multiple charged ions which led to differences of m/z of only 2 (m/z 720 and 722 respectively for natural isotope abundance and $^{13}\text{C}_4$ labelled pentamers). In addition, the natural occurrence of heavy isotope is especially relevant with larger molecules emphasizing the need to obtain ^{13}C -labelled material with a DP higher than four with more than four labeled carbons. In the current study, only DP1-4 ^{13}C -labelled reference compounds were available as internal standards for method development. Although financially constraining, ideal ^{13}C -internal standards should be labelled on each oligomer monomeric unit. This approach would avoid the dilution of response between labelled and unlabelled fragments.

Optimization of and chromatographic conditions. Liquid chromatography was implemented to separate cocoa flavanol and procyanidin oligomers by DP using a column featuring a diol-modified sorbent. The official method of analysis relies on the separation of procyanidins under HILIC conditions. These

conditions involve a high flow rate (1 mL/min) and a high concentration of acetic acid (2 %) in the mobile phase and result in ion suppression that can be counteracted, in part, by using a post-column additive such as ammonium carbonate¹⁶. To limit the ion suppression phenomena, the method optimization in the current study focused on reducing the amount of acid entering the electrospray source. Use of a mobile phase containing 0.5 % formic acid resulted in a slight decrease in response but a 10-fold improvement in the signal to noise ratio (S/N). In addition, MS² detection allows for a slight overlap of signals leading to shorter run time than observed with fluorescence detection without compromising analytical performance. With these conditions, the flow rate was decreased to 0.3 mL/min and the gradient shortened to 10 min (when compared to 1 mL/min and 16 min for AOAC2020.05). A typical chromatogram is illustrated in Fig. 4.

Sample preparation development; accuracy by design. Sample preparation was optimized for seven model matrices, two of which were cocoa-based. These matrices were selected to provide a versatile sample preparation process that can accommodate different sample formats and, at the same time, provide insights in the quantification of flavanols and procyanidins in sources other than cocoa. Detailed preparation for each of the seven matrices studied is outlined in Table S1. The evaluation of sample preparation began with the evaluation of the recovery parameter (comparison of content measured in sample spiked before and after extraction). Results are shown in Fig. 5 and highlight that there is no significant loss under the sample preparation conditions used.

Matrix effects were evaluated by comparing the responses of CF in NIST RM 8403 and ¹³C labelled synthetic epicatechin and B-type procyanidins in a matrix solution and a standard solution. For matrices containing endogenous levels of CF, target response was corrected for endogenous content. Matrix effects show two main trends (Fig. 5). First, responses were different across degree of polymerization which show that the use of structural analogues as internal standards cannot achieve accurate quantitative analysis of CF by HPLC-MS². Second, ¹³C-labelled epicatechin and procyanidins showed comparable response to those isolated from cocoa. Thus ¹³C-labelled flavanol and procyanidins as shown in Fig. 1 can be used as internal standards alone or in combination with matrix match to compensate matrix effects.

Importantly, matrix effects were inconsistent across samples and across origin for a given matrices. For example, the matrix effect with three different brands of dark chocolate were evaluated at 47, 56 and 86 %, indicating that the calibration curves built with one sample matrix blank would not be applicable to the two others. In this context, two approaches are suitable to achieve accurate quantitative analysis. First, the matrix match approach does not involve the use of expensive ¹³C-labelled internal standards, but is less robust to interferences. The matrix match can be considered a reliable approach to achieve accurate measurement with the caveat that the matrix blank used to build calibration curve sample must be representative of the sample under study. This highlights the need to prepare multiple calibration curves when multiple types of samples are analyzed, even though these samples might, for example, all be dark chocolates. The second approach involves the use of stable isotope-labelled internal standards which is

the most efficient and remains necessary when blank matrices are not available. To minimize the use of expensive ^{13}C -labelled procyanidin internal standards, the matrix match approach was adopted when a blank matrix was available, while a ^{13}C -labelled internal standards was used for cocoa samples for which a blank matrix was not available.

Method validation. Method validation focused on determining accuracy, precision, sensitivity and selectivity parameters. Due to the availability of internal standards, experiments focused on CFs DP1-4. The quantification of CFs DP1-4 provides a significant input into material composition as it represent approximately 70 % of total CFs (DP1-7) and covers the DP1-2 CFs for which dietary absorption in humans has been demonstrated ¹⁷.

The first parameter evaluated was the method linearity and sensitivity. Method sensitivity is shown in Table S3 with the LOQ ranging from 10 to 50 ng/mL. These limits were determined as a $S/N \geq 10$ were systematically below the lowest point of the calibration curve. Linearity was demonstrated by examining the coefficient of correlation of the calibration curve. A quadratic fit was used because a slight detector saturation was observed with DP1-2. This is attributed to higher concentration in the secondary standard used and a higher detector response than that observed for DP3-4. Regardless, coefficients of determination were systematically higher or equal to 0.99.

Precision and accuracy were assessed using a spike and recovery approach (Table 2). For each model matrix with an available blank, a stock solution was prepared and spiked with three different level of NIST RM 8403. The relative standard deviation across triplicate preparations determined precision, while the comparison of the measured value to the spiked amount estimated accuracy. For the two matrices containing endogenous procyanidins (cocoa powder and baking chocolate), four levels were prepared and only precision could be reported in the blank samples. As expected, precision improved as concentration increased. As shown by Table 2, intraday precision (%RSD) of up to 22.1% in LOQ and blank levels, while the highest %RSD was 5.7% at MQC and HQC levels. Similarly, accuracy ranged from 90.9 to 125.4% at LQC level and 91.1 to 111.5% at MQC and HQC. Interday precision showed similar trend with %RSD up to 20.0% at LQC level and 11.2 % for MQC and HQC levels (interday precision data is available in supplementary information Table S4). These performances suggest that the HPLC-MS² method develop can accurately and precisely measure flavanols and procyanidins.

Table 2

Percentage accuracy (%Acc) and intraday precision (%RSD) parameters for cocoa powder and baking chocolate using ^{13}C labeled internal standards and for whole milk powder, whey protein, corn starch, soy flour and wheat flour using matrix match calibration. Low, Middle and High Quality Check (LQC, MQC and HQC), not analysed (na).

		DP1		DP2		DP3		DP4	
		%Acc	%RSD	%Acc	%RSD	%Acc	%RSD	%Acc	%RSD
Whole Milk	LQC	115.1	9.5	115.6	7.7	114.0	4.7	125.4	9.8
	MQC	108.7	2.3	109.5	1.8	106.7	4.4	108.7	4.7
	HQC	103.3	4.2	102.2	1.0	101.0	2.7	107.6	3.2
Wheat Flour	LQC	92.5	9.4	94.0	3.8	99.4	2.6	92.4	4.2
	MQC	109.5	3.8	103.2	1.6	105.6	1.7	102.7	1.8
	HQC	111.5	3.1	105.7	2.1	107.2	0.8	105.0	3.6
Corn Starch	LQC	91.5	4.8	97.3	3.1	95.2	4.3	101.7	3.9
	MQC	103.6	2.0	107.2	1.0	105.2	4.9	107.3	3.3
	HQC	97.0	3.5	102.0	2.6	100.7	1.6	104.1	2.7
Whey Protein	LQC	90.9	8.8	97.2	2.2	104.1	3.7	105.1	4.6
	MQC	99.4	4.8	97.7	1.2	100.6	4.7	104.4	2.9
	HQC	99.4	2.7	100.0	1.1	101.1	4.1	104.1	2.6
Soy Flour	LQC	96.4	2.2	101.4	3.2	104.0	3.3	99.3	2.8
	MQC	103.1	2.2	105.2	1.7	104.3	3.4	104.7	2.4
	HQC	101.7	2.6	105.8	1.8	103.8	2.6	104.8	3.3
Cocoa powder	blank	na	5.1	na	22.1	na	21.9	na	19.1
	LQC	101.9	8.1	103.9	10.9	99.3	4.9	100.9	5.6
	MQC	100.4	5.7	101.4	3.5	96.6	1.3	100.8	2.7
	HQC	100.3	3.6	100.7	1.4	92.5	2.0	102.1	2.1
Baking chocolate	Blank	na	5.0	na	5.1	na	5.9	na	5.8
	LQC	104.0	5.3	95.2	4.9	104.3	5.5	99.5	1.9
	MQC	107.0	1.6	95.2	2.0	105.6	2.2	108.3	2.6
	HQC	101.2	1.0	91.1	2.1	104.4	0.8	105.5	5.7

Method selectivity was investigated for two potential causes of interferences. The first source of interference investigated was between procyanidins with a different DP. With the formation of multiple charged ions, interferences are observed between targets, for example DP2 and DP4 show respective interferences with the double charged DP4 and double charged DP8. In this context, the separation of interfering oligomers by HPLC was essential to achieve the desired selectivity. The second type of interference come from matrix components or other types of procyanidins that are not targeted by the method. A-type procyanidins are present in many botanicals, including cranberry and cinnamon. A- and B-type procyanidins share different CID patterns that can be leverage to develop the measurement of B-type procyanidins independently from A-type procyanidins and vice-versa. To verify the selectivity of the method toward B-type procyanidins which are the only form identified in cocoa, procyanidins A1 and A2 (see structure in Fig. 3) were analysed and no signal was detected, confirmin that cocoa is not a source of these specific procyanidins. Procyanidin B5 was also analyzed and the concentration was underestimated when compared to CF DP2 and isolated procyanidin B2 (Fig. 3). This suggested that the selectivity of this methodology was specific to C4→C8 B-type procyanidins, but C4→C6 B-type procyanidins could also contribute to the signal. This shows the limitation of quantative MS² detection of procyanidins under the chromatographic conditions selected for this method. While reliable results can be achieved to differentiate A- and B-type linked species, MS-MS detection does not allow discrimination between B-type procyanidins of the same DP. In order to achieve accurate results in the quantification of procyanidins in a given botanical, a reference material that matches the structural diversity of the targeted procyanidins in the sample represents a better alternative than using individual procyanidins as analytical standards under the chromatographic conditions of this method (see Fig. 4). In this context, NIST RM 8403 was investigated as a secondary standard mixture. This material consists of a highly characterized cocoa extract that is already used as a secondary standard for the quantification of CF using HPLC-FLD methods ¹⁵. Primary standards isolated from cocoa were used in a standard addition experiment to determined NIST RM 8403. Table S5 summarizes the concentration assigned by NIST on RM 8403 using HPLC-FLD ¹⁸ and the estimates determined using HPLC-MS² and standard addition of primary standards. Relative differences ranged from 2.1 to 4.2 %, and < 3 % for the sum of DP1-4. For three of the four oligomers, differences between the content determined and the value ascribed to NIST RM 8403 were below the standard deviation observed on the triplicate measurement. This suggests that NIST RM 8403 is an appropriate secondary standard calibrant for the determination of CF with degree of polymerization up to four by HPLC-MS². The NIST RM 8403 could be considered for the estimation of putative procyanidin concentrations in other botanicals with the caveats that the differences between procyanidin structures in the RM 8403 (cocoa) and the botanical of interest will lead to accuracy biases.

Stability of the samples was evaluated at 5°C for autosampler and -20°C for freezer temperature. Internal ¹³C-labelled standards were not used for these measurements as they likely degrade at the same pace than the targets and would bias the evaluation of stability. Results are shown in the Supporting Information (Figure S2) for DP1-4 over 4 days. With the exception of DP3 in cocoa powder, samples were stable for 48 hours after preparation for the two temperatures studied. DP3 in cocoa powder showed a significant loss at both temperature after 48 h but was stable for 24 h.

Comparison to AOAC official method of analysis (AOAC2020.05). The method developed provides B-type specific quantitative analysis DP1-4 CFs. Because the HPLC-MS² method was subjected to matrix effects than can impact on recovery, when possible, it is critical to evaluate method accuracy through side-by-side comparison with an existing known reliable testing method, such as AOAC official methods of analysis (AOAC2020.05 for CF).¹⁹ To establish this comparison, a set of 26 samples, covering a wide range of concentration (3 to 540 mg/g) was analyzed using both methods. Figure 6 shows a Bland-Altman plot of the relative difference between DP1-4 determined with the HPLC-MS² method and the AOAC accredited HPLC-FLD protocol. Similar plots are available in the Supplementary Information for the individual DPs (Figure S3) and results are presented in Table S6. The relative difference average was 4.5%, indicating good agreement between the HPLC-MS² and accredited testing methods. This slight underestimation of DP1-4 content can be attributed to the higher selectivity of MS² detection. These results show that the results obtained by HPLC-MS² are comparable to those determined through accredited testing and therefore demonstrate appropriate accuracy performance. Because of the scarcity of ¹³C-labelled procyanidins, the higher precision performances of fluorescence detection and the cost of instrumentation, HPLC-FLD would be the method of choice for routine analysis of CFs. However, when higher selectivity is sought and CF measurement is required to be specific to B-type procyanidins, HPLC-MS² is the method of choice. Finally, HPLC-MS² testing of CFs could be used to resolve disputes between manufacturers and/or with regulatory bodies, or when the authenticity of a cocoa product needed to be verified by providing a more selective alternative to the existing methods relying on fluorescence detection.

Conclusions

HPLC-MS² represents a more selective quantification tool for cocoa flavanols and procyanidins than the HPLC-FLD method currently used for routine testing. Importantly, the MS-based method was shown to be in good agreement with the FLD-based method for the predominate DP present in cocoa (DP 1–4), enabling the methods to be used in a complementary fashion. The performances associated with MS² detection require the use of ¹³C-labelled internal standard and the availability of standard or reference material for method calibration that are representative of the structural diversity of the targeted procyanidins. In the case of cocoa, the now commercially available NIST RM 8403 is a suitable secondary standard that can serve the determination of cocoa based samples. Together, this work demonstrates that HPLC-MS² can be used to supplement routine testing by AOAC2020.05 when fluorescence detection data needs to be verified by a higher/more selective method procedure or when fluorescence detection cannot accommodate analysis of CF in complex matrices.

Furthermore, this method was capable of specifically quantifying B-type procyanidins even in the presence of A-type procyanidins of similar size, thus demonstrating a higher selectivity than fluorescent detection. The quantitation of DP1-4 provides an insight on approximately 60–70% of the cocoa flavanol and procyanidin content. More importantly, DP1-4 content includes flavanol monomer and procyanidin dimer which are the main compounds being absorbed and showing bioactivity. Future work should

include the development of ^{13}C labelled procyanidin for higher degree of polymerization (pentamer and larger) as these compounds have also been suggested to have putative health benefits^{20,21}. The access to synthetic ^{13}C labelled standards, NIST reference material and pure standards isolated from cocoa were key enablers of this work. While the NIST RM 8403 was demonstrated to be an adequate secondary standard for the determination of flavanol and procyanidin in cocoa. In this context, NIST RM 8403 could be considered as a potential reference material for the initial estimation of procyanidin concentrations in other botanicals, particularly those mainly containing B-type procyanidins. The work presented in this manuscript focuses on cocoa and method performances were evaluated for CF determination; however, learnings associated to the development of selective method for cocoa flavanol and procyanidins could be leveraged to study other botanicals. Flavanol and procyanidins are found in variety of food and can be consumed in healthy plant-rich diet. The development of such methods remains critical to further understand flavanol and procyanidin distribution in the diet and to support investigations aiming at understanding the nutritional relevance of this group of polyphenol bioactives.

Material And Methods

Material and reagents. HPLC grade solvents, (-)-epicatechin [(-)EC], procyanidins A1, A2, B2 and B5 standards were purchased from Sigma-Aldrich (Saint-Louis, MO, USA) and referred to as synthetic standard. (-)-[2,3,4- $^{13}\text{C}_3$]Epicatechin ($^{13}\text{C}_3$ -(-)EC) was purchased from Cambridge Isotope Laboratories (Cambridge Isotope Laboratories, Inc Tewksbury, MA, USA). Stable isotope labelled synthetic standard for procyanidins - procyanidin B2, ($^{13}\text{C}_4$ -DP2); procyanidin C1, ($^{13}\text{C}_4$ -DP3) and cinnamtannin A2, ($^{13}\text{C}_4$ -DP4) were purchased from Analyticon Discovery with purity of $\geq 90\%$ and no residual trace of unlabeled procyanidins. Carbon positions labelled with ^{13}C are shown in Fig. 1. CF oligomeric fractions including DP₂₋₇ isolated from the seeds of *Theobroma cacao* L. by Mars Wrigley Confectionary (Mars Inc, Hackettstown, NJ, USA), and purified and characterized (purities $> 94\%$) by Analyticon Discovery (Analytical Discovery GmbH, Potsdam, Germany) served as CF oligomer primary standards as previously reported²².

CF extract reference material (RM 8403)²³, baking chocolate reference material (RM 2384), whole milk powder (RM 1549a), and soy flour (RM 3234) were acquired from the National Institute of Standard and Technology (NIST, US Dept. of Commerce, Gaithersburg, MD, USA). Corn starch, wheat flour, and whey protein were purchased at a local grocery store. Cocoa powder was supplied by Mars Symbioscience (Mars Inc, Germantown, MD, USA).

Sample preparation. Seven matrices were selected to represent different foodstuff composition. The distribution of the composition of these food matrices are presented in Figure S1. Sample preparation was optimized for each of the seven foodstuff models using a combination of processing steps including defatting, solid/liquid extraction, protein precipitation and SPE clean-up which are summarized in Table S1 in the on-line Supplementary Information.

Defatting of high fat matrices. Fat removal was performed for matrices with fat content above 10 % by weight using and solid liquid extraction with hexane. Defatting was carried out by mixing 5 g of test material with 45 mL of hexane, sonicating at 50°C, followed by centrifugation for 5 min at 1700 rcf. The hexane wash was decanted and the process repeated until the supernatant became clear. The hexane washings were combined and evaporated overnight at room temperature. The dry hexane residue was weighed to determine % fat content and enable fat correction of results. The defatted solid prepared for analysis.

Solid/liquid extraction. CFs were extracted from solid samples with acetone:water:acetic acid (70/30/1, v/v) (AWAA). The mixture was vortexed, sonicated 5 min at 50°C, and centrifuged for 5 min at 1700 rcf. The supernatant was either filtered prior to analysis or subjected to SPE purification.

Protein precipitation. Water was added to samples which were vortexed, acetone:acetic acid (99.5/0.5, v/v) was added prior to sonication at 50°C and incubation at -20°C for 20 min to allow complete protein precipitation. Samples were then centrifuged at 1700 rcf at room temperature for 5 min. The supernatant was either filtered prior to analysis or subjected to SPE purification.

SPE purification. Solid phase extraction use a mixed mode cation exchange cartridge (Oasis PRiME MCX 6 cc 150 mg) (Waters Corporation, Milford, MA, USA). A 1 mL volume of AWAA was used to condition the cartridge until ca. 2 mm remained on top of the sorbent. The cartridge was loaded with 2.5 mL of sample supernatant then as eluted until 2 mm remained on top of the sorbent. The sorbent was then washed with 12 mL AWAA which was collected and made up to a 25 mL volume with AWAA prior to analysis.

Matrix Effect and Recovery. Matrix effect and recovery parameters were used to estimate sample preparation extraction and clean-up performances. Matrix effect and recovery were estimated using the a solution of standard, a matrix blank, a matrix spike (before extraction) and a matrix spike (after extraction). Matrix effect was calculated as the ratio of the difference between the area response (HPLC-MS²) of matrix blank and matrix spiked after extraction to that of the standard solution. Recovery was estimated as the ratio of the response of the matrix spiked before and after extraction. Sample preparation was tailored to the composition of each matrix (see Table S1).

HPLC-MS² optimization. The accurate analysis CFs by HPLC with MS² detection is dependent upon the use of a chromatographic mobile phase without interferences from compounds with similar *m/z* ions to those produced by the compounds of interest. Full MS scans of the reference material (RM 8403) and primary standards were performed to identify *m/z* of parent compounds and optimal cone voltage conditions for CFs with different degrees of polymerization. The reference material and the primary standards were isolated from cocoa and, therefore, a C4→C8 B-type linkage was expected to be the predominant structural feature of the targeted molecule. Cone voltage was evaluated from 10–80 V with an increment of 5 V and *m/z* scanned from 50 to 2000 Da. Daughter scans were then acquired for each target. Fragments were selected for their contribution to sensitive and selective detection. Collision energies were optimized from 10 to 80 V. To select the best compromise between sensitivity and

selectivity, transitions that were unique to the targeted procyanidin multiple reaction monitoring (MRM) transitions were selected to achieve resolution against adjacent analytes in priority. MS² parameters are summarized in Table 1.

HPLC-MS² analysis. A Water Acquity H-class liquid chromatograph linked to a tandem mass spectrometer (Waters Xevo TQS micro) with an electrospray source operating in negative mode was used for flavanol and procyanidin analysis. Chromatographic separations used a Waters Torus Diol Column (2.1 x100 mm, 1.7 µm particle size, 130 Å pore size) fitted with an in-line filter. Column and autosampler temperatures were set to 50°C and 5°C, respectively. Samples, 2 µL, were injected and separated by a binary gradient with mobile phase A (acetonitrile:formic acid; 99.5:0.5) and mobile phase B (methanol:water:formic acid; 97:3:0.5). The solvent gradient at 0.3 mL/min was 0.0–0.4 min, 0%B, 3.0 min 45%B, 5.5 min, 95%B, 6.5 min 95%B, 6.6 min 0%B, and 10.0 min 0%B).

Negative mode electrospray ionization settings were as follows: desolvation gas flow was at 800 L/hr, desolvation temperature was 500°C, cone gas flow was 100 L/hr, capillary voltage was at 3.2 kV, quadrupole low and high mass resolutions were lowered for the first and third quadrupole (LM1 resolution was set at 9.2, HM1 resolution was set at 12.0, LM2 resolution was set at 9.2, and HM2 resolution was set at 12.0) to accommodate the detection of multiple charged ions showing wider signal with low resolution detector. MRM detection conditions are described for DP1-7 in Table 1. However, due to accessibility to ¹³C labelled material, quantitative method development was only possible for DP1-4.

Calibration. Cocoa extract reference material RM 8403 has been developed for the purpose of calibrating HPLC-FLD instruments used in AOAC Official Method of Analysis 2020.05. In this study, we used RM 8403 to calibrate a HPLC-MS² instrument by preparing a serial dilution of NIST RM 8403. The stock solution was prepared by dissolving 40 mg of RM 8403 in a 50 mL flask with acetone:water:acetic acid (AWAA 70:30:1). This solution was diluted ten times to provide working standard #7. Working standard #7 was then diluted by pipetting 1.25, 2.5 and 5 mL in 10 mL volumetric flasks to obtain working standards 4–6 and 0.4, 0.8 and 1.5 mL in 25 mL volumetric flasks to obtain working standards 1–3. For cocoa samples, 1 mL of working standards 1–6 was transferred to autosampler vial and 10 µL of ¹³C internal standard solution (50 µg/mL) was added. Calibration curves were built for DP1-4 using the relative response of each target to its respective ¹³C internal standard, a 1/χ weighing function and a quadratic model. For samples with blank matrix available, a calibration curve was built in a similar manner, but with the use of ¹³C internal standard. Instead, a matrix match approach was preferred as a more cost-efficient option.

Method validation. Method accuracy was determined through a spike and recovery approach at three levels, each prepared in triplicate. LQC (or low quality check) was the lowest level and was within 3 times the lowest level of the calibration curve. The intermediate level, MQC (or medium quality check), was placed in the middle of the calibration curve. The high quality check, HQC, had a concentration between the second to highest and highest level of the calibration curve. A solution of matrix was prepared at a concentration 4 times higher than what is recommended in Table S1, and 2.5 mL were transferred in 10

mL volumetric flasks. A solution of cocoa extract reference material was spiked at three levels and the volume adjusted to 10 mL. Accuracy was determined as the ratio between the measured concentration to spiked concentration. For cocoa powder and baking chocolate, the measured concentration was corrected for endogenous CF measured in unspiked samples.

Precision was assessed at three levels (four levels for matrices that contain endogenous CFs) and defined as the relative standard deviation (%RSD) across triplicate preparation within a single sequence (intraday) and three set of triplicate preparations analysed in three consecutive days (interday).

Linearity was determined by the coefficient of determination (r^2) higher or equal to 0.99. A quadratic fit was necessary due to the disparity in concentration and response across the degree of polymerization of the procyanidins. In these conditions, a slight saturation of the signal was observed at the highest concentration and led to using a quadratic model to fit the response=f(concentration) curve.

Selectivity was determined by analyzing other types of procyanidins. Cocoa procyanidins are predominantly B-type while other botanicals can contain A-type procyanidins or a mix of both A and B procyanidins. Commercially-available A-type procyanidins (procyanidin A1 and A2) were analyzed and showed no signal.

Stability was evaluated for the seven matrices over four days at autosampler (5°C) and at freezer (-20°C) temperatures (Table S2). Each sample was determined using a calibration curve solution prepared on the day of analysis. Percentage recoveries were calculated as the ratio of the content determined to the content determined on day 1, for DP1-4, for each matrix over a 3 day period.

Cross-validation. To estimate method reliability, performances were compared to those of the recently accredited testing (AOAC2020.05). Twenty six cocoa-based samples were analysed using the HPLC-MS² method and the fluorescence-based protocol detailed in AOAC 2020.05. Sample matrices included a wide-range of commercially-available cocoa-based ingredients including cocoa powder, dark chocolate, baking chocolate, dietary supplement drink mixes, dietary supplement capsules and dietary supplement cocoa extract ingredients. These samples had CF contents ranging from ca. 3 to 500 mg/g (DP1-7 per AOAC2020.05). Given the limited number of de novo radiolabeled standards, DP1-4 contents measured and compared across the two methods. Method differences were assessed by modeling CF content determined by HPLC-MS² as a function of CF content determined with HPLC-FLD.

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Figures

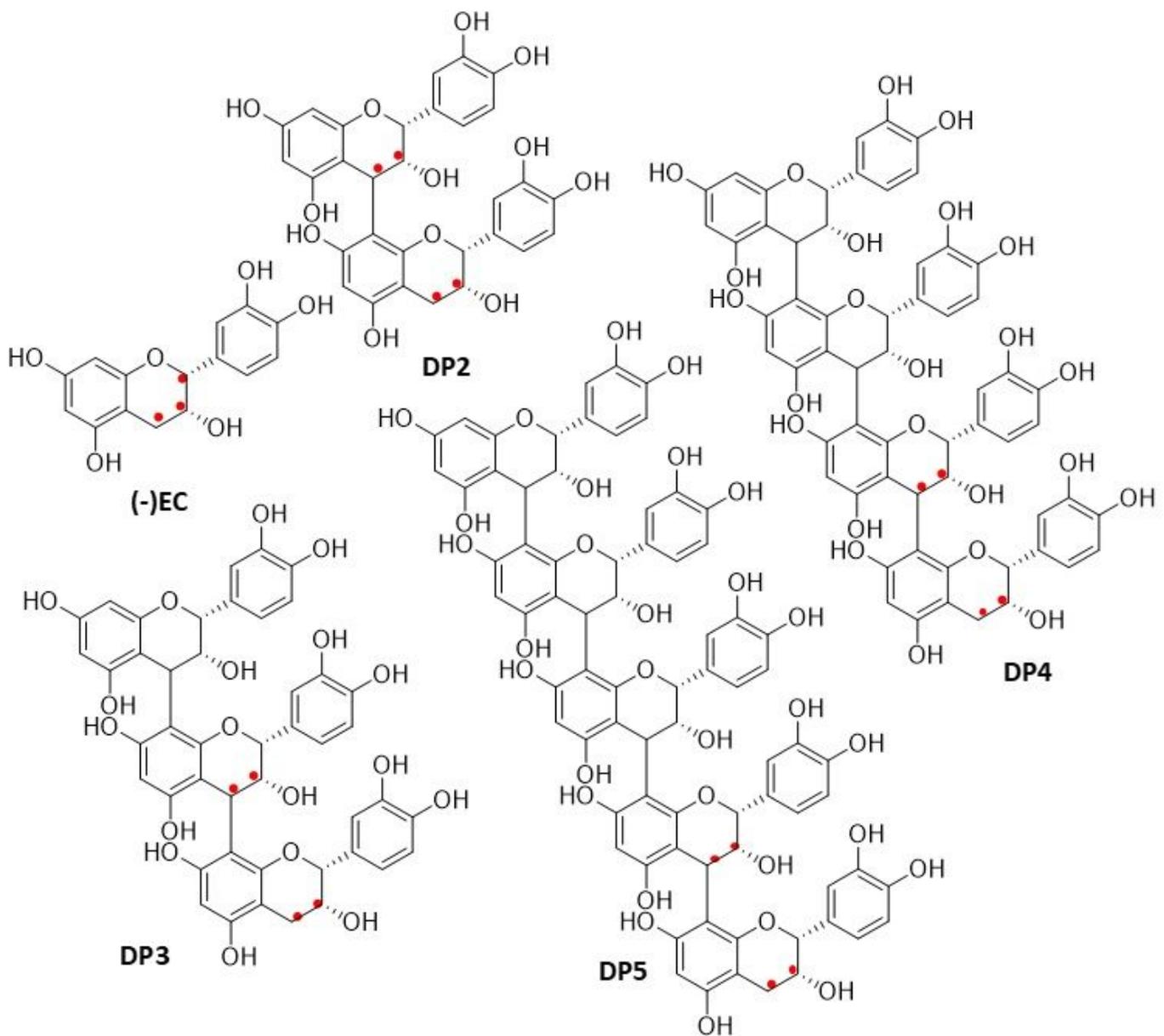


Figure 1

Structure of (-)-epicatechin and (-)-epicatechin oligomers (C4→C8 B-type procyanidin) with degree of polymerization up to five (DP2-5), red dots represent carbon that are labelled with ¹³C on synthetic procyanidin standards.

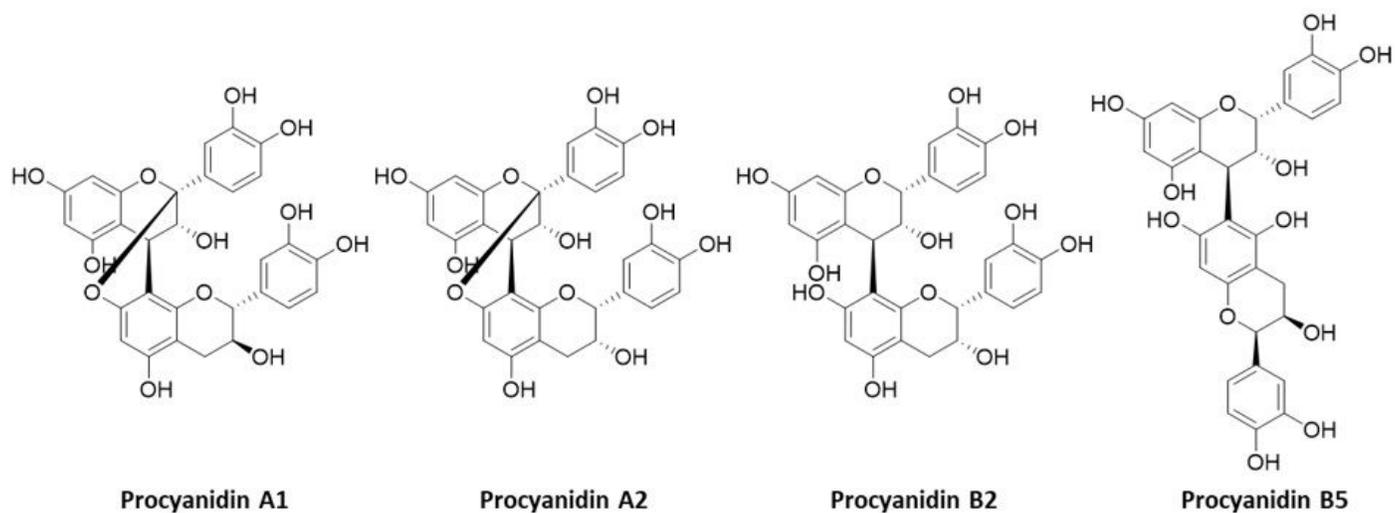


Figure 2

Structure of procyanidin A1, A2, B2 and B5 used for the evaluation of specificity.

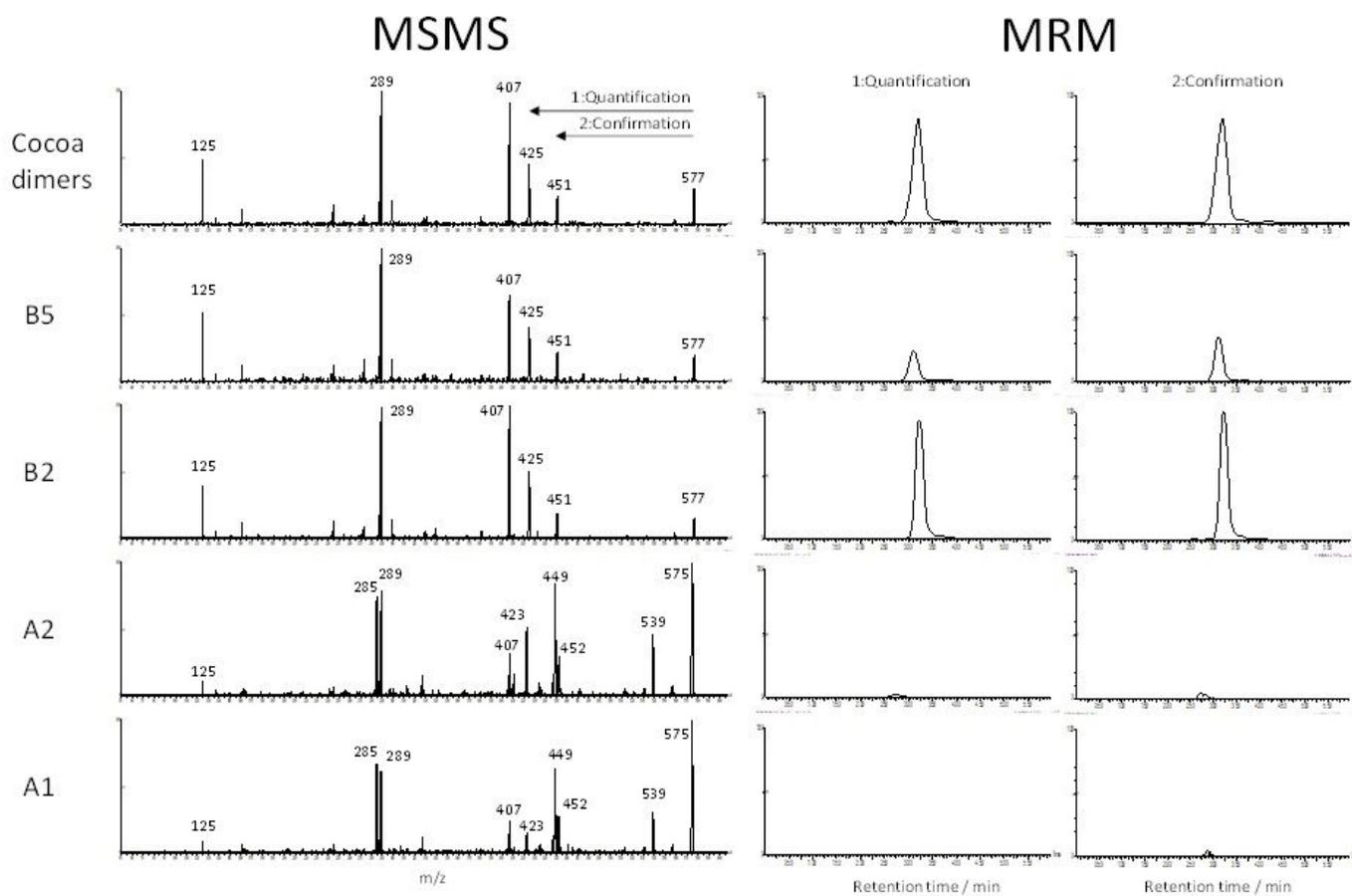


Figure 3

Left: MS-MS collision induced dissociation with the MRM transitions used for quantification (577>407) and confirmation (577>425) of b-type procyanidin dimer. Right: HPLC-MS2 (MRM) of cocoa procyanidin with a DP2, procyanidin B5, procyanidin B2, procyanidin A2 and procyanidin A1 (top to bottom). Samples were all prepared at 10 µg/mL.

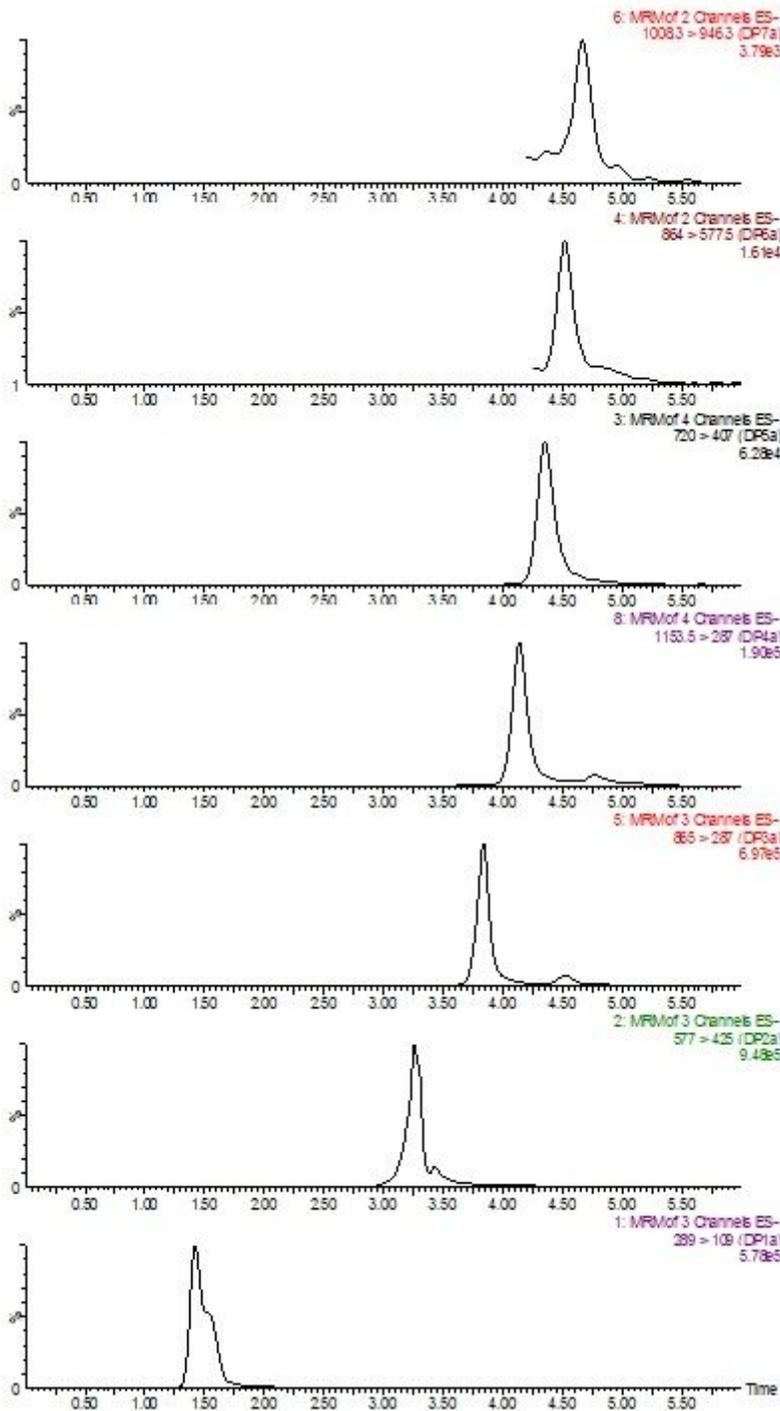


Figure 4

HPLC-MS2 extracted ion chromatogram of cocoa flavanols and procyanidins with degree of polymerization from 1 to 7 (bottom to top) in baking chocolate at endogenous concentrations.

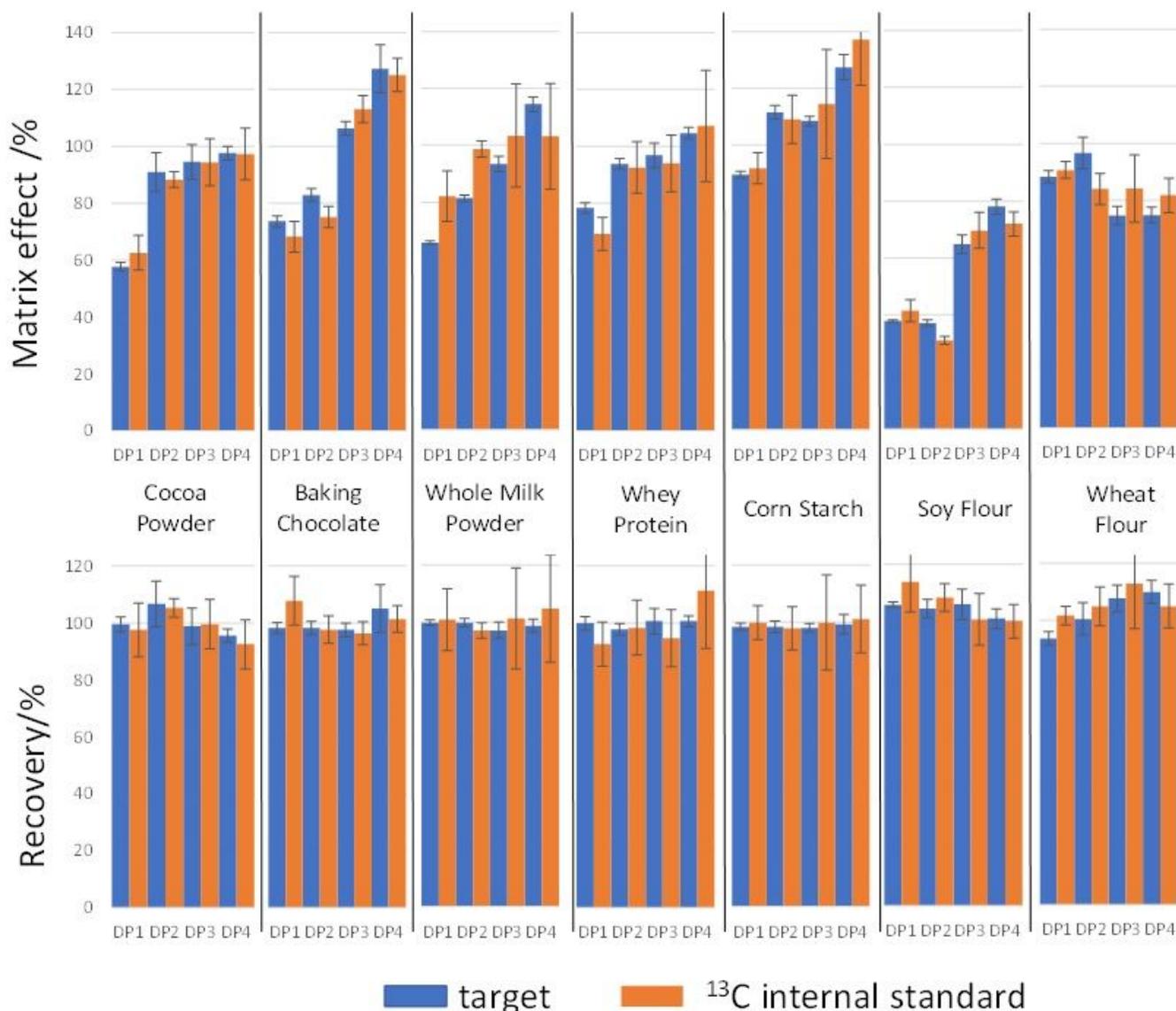


Figure 5

Matrix effect and recovery parameters for DP1-4 targets and respective ¹³C labelled internal standard in cocoa powder, baking chocolate, whole milk powder, whey protein, corn starch, soy flour and wheat flour. Blue and orange bars represent natural isotop abundance flavanol and procyanidins and ¹³C labelled (-)-epicatechin and epicatechin oligomers respectively. Error bars show the standard deviation for five replicates. Recovery (in %) were determined by comparing signal area in sample spiked before and after extraction. Matrix effect (in %) were determined by comparing signal area in sample solution spiked after extraction and standard solution.

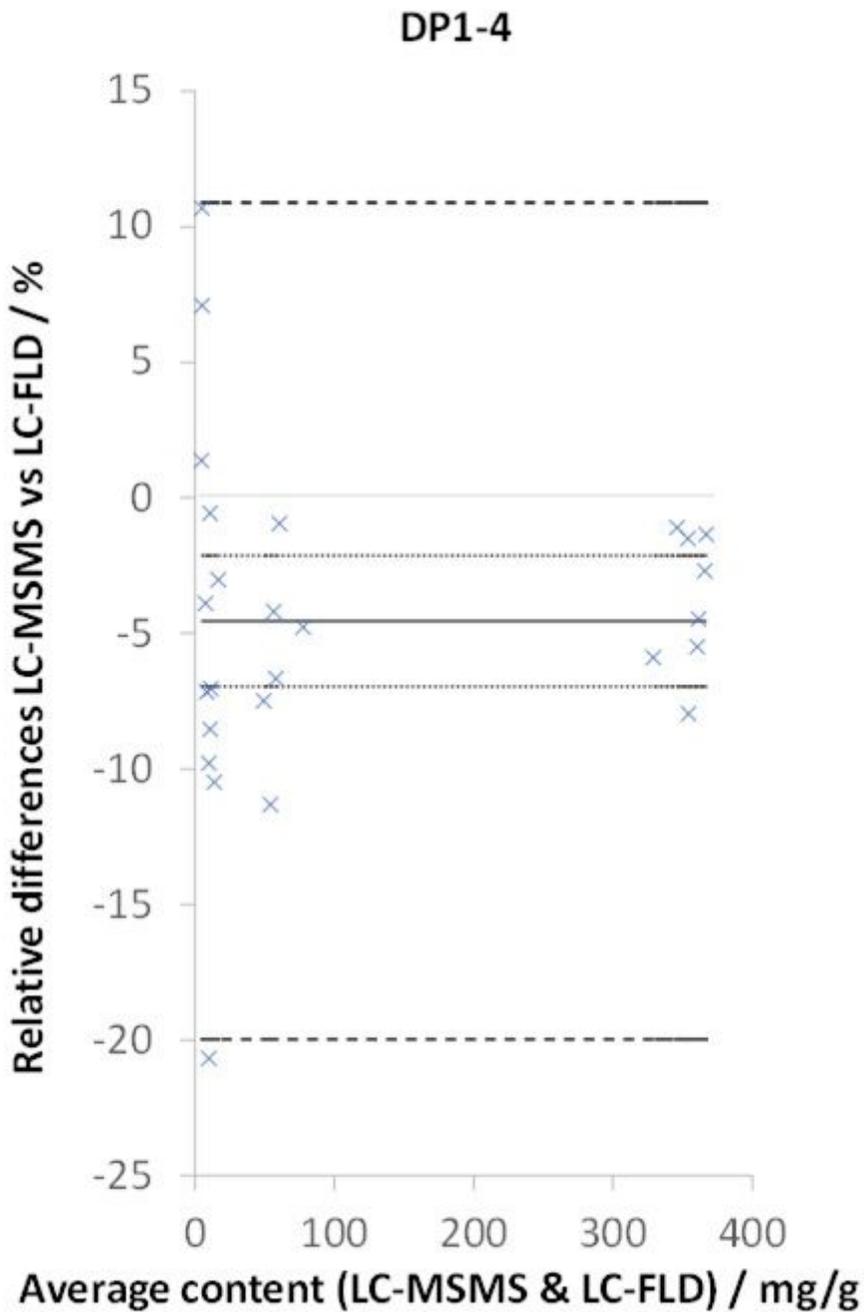


Figure 6

Bland-Altman plot of cocoa flavanol and procyanidin (DP1-4) relative difference between HPLC-MS2 in % testing and accredited HPLC-fluorescence detection (HPLC-FLD) testing as function of average DP1-4 content determined by both methods in mg/g. Solid black line represents the average relative difference between the two methods, the dotted line represent the 95% confidence on the average relative difference and the dashed black line represents the 95% confidence interval on a single estimate.

Supplementary Files

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